(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 October 2004 (07.10.2004)

PCT

(10) International Publication Number
WO 2004/085478 A2

(51) International Patent Classification[7]: C07K 19/00, 14/47, 14/475, 14/525, 14/52, 14/54, 14/705, 14/715

(21) International Application Number: PCT/EP2004/003239

(22) International Filing Date: 26 March 2004 (26.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
03006949.6 26 March 2003 (26.03.2003) EP

(71) Applicant *(for all designated States except US)*: APOGENIX BIOTECHNOLOGY AG [DE/DE]; Im Neuenheimer Feld 581, 69120 Heidelberg (DE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: WALCZAK, Henning [RU/DE]; Keplerstrasse 17, 69120 Heidelberg (DE).

(74) Agents: WEICKMANN, Franz, Albert et al.; Weickmann & Weickmann, Postfach 860 820, 81635 München (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/085478 A2

(54) Title: IMPROVED FC FUSION PROTEINS

(57) Abstract: The invention relates to fusion proteins comprising at least a first domain and a second domain selected from a constant Fc immunoglobulin domain.

Improved Fc Fusion Proteins

Description

The invention relates to fusion proteins comprising at least a biologically active polypeptide domain and a second domain selected from a constant immunoglobulin domain.

Fusion proteins comprising an immunoglobulin heavy and/or light chain dimer or an immunoglobulin heavy and/or light chain tetramer, in which an amino acid sequence of a ligand-binding partner which is a receptor, a carrier protein, a hormone, a growth factor or an enzyme, is substituted for the variable region of at least one immunoglobulin chain, are described in EP-A-0 526 452. A fusion protein comprising the extra cellular domain of the death receptor CD95 (APO-1; Fas) fused to an immunoglobulin Fc fragment is described in WO 95/27735. N-terminally truncated derivatives of the APO-1 molecule optionally fused to immunoglobulin Fc fragments are disclosed in EP-A-0 965 637. A fusion protein consisting of soluble IL-15Rα and Fc fragments is disclosed in WO 98/36768. A fusion protein consisting of an antagonist IL-15 mutant and an Fc IgG2a fragment is disclosed by Kim et al. (*J. Immunol.* 160 (1998), 5742-5748). These documents are incorporated herein by reference.

Although it has been shown that fusion proteins as described above have high biological activity *in vitro* and *in vivo,* there are concerns with regard to the immunogenic potential of such molecules since there is a fusion region between two protein domains of different origin comprising a non-naturally ocurring amino sequence which may elicit an undesired immune response in an organism to which the fusion protein is administered.

WO 02/066514 describes artificial fusion proteins having a reduced immunogenicity compared to the parent non-modified molecule when exposed to a species *in vivo.* These proteins essentially consist of an

immunoglobulin molecule or a fragment thereof covalently fused via its C-terminus to the N-terminus of a biologically active non-immunoglobulin molecule, preferably a polypeptide or protein or a biologically active fragment thereof. The molecules have amino acid sequences which are altered in one or more amino acid residue positions but, in principle, have the same biological activity as compared with the non-altered molecules. The changes are made in regions of the molecules which are identified as T-cell epitopes, which contribute to an immune reaction in a living host. A disadvantage of this procedure, however, is that not all epitopes, particularly not B-cell epitopes, can be reliably eliminated. Furthermore, the introduction of non-naturally occurring amino acid sequences can lead to the generation of neo-epitopes.

Thus, it was an object of the present invention to provide fusion proteins with at least two domains of different origin having a reduced immunogenic potential.

Thus, the present invention relates to a fusion protein comprising
(i) at least one first domain comprising a biologically active polypeptide and
(ii) a heterologous second domain comprising at least a portion of a constant immunoglobulin domain,
wherein there is at least one amino acid overlap between the first domain and the second domain in the fusion region.

The fusion protein may be a monomeric protein or a multimeric protein, e.g. a dimeric or tetrameric protein, which may be formed by multimerisation via the constant immunoglobulin domain.

According to the present invention, the design of a fusion protein comprises i) the selection of at least one first domain and a second domain which is heterologous to the first domain and ii) the selection of at least one terminal amino acid which is common to the first and the second domain, e.g. the last amino acid(s) of the first domain is (are) selected such that they are identical

with the first amino acid(s) of the second domain. Preferably, the overlap has a length of one, two or three amino acids. Thus, a fusion protein is obtained which is free from a non-naturally occurring transition between the last amino acid of one domain and the first amino acid of another domain.

In an embodiment of the invention, the first domain(s) is (are) located at the N-terminus of the fusion protein, whereas the second domain is located at the C-terminus. Thus, in this embodiment, at least one carboxy terminal amino acid of a first domain overlaps with at least one amino terminal acid of the second domain.

In a further embodiment the second domain is located at the N-terminus of the fusion protein and the first domain(s) is (are) located at the C-terminus. Thus, in this embodiment, at least one carboxy terminal amino acid of the second domain overlaps with at least one amino terminal acid of a first domain.

In cases where the fusion protein comprises more than one, e.g. two or three, first domains, these domains are preferably located sequentially at the N-terminus or the C-terminus of the fusion protein and the second domain at the C-terminus or at the N-terminus, respectively. It should be noted that the first domains in such proteins may be the same or different. Transitions between individual first domains are preferably designed such that there is also at least one amino acid overlap (and thus not a non-naturally occurring transition between the last amino acid of one domain and the first amino acid of the other domain) between the individual first domains. Fusion proteins comprising multiple first domains are disclosed in WO 00/18932 which is incorporated herein by reference.

The first domain of the fusion protein comprises a biologically active polypeptide, i.e. a polypeptide which is capable of interacting with, e.g. binding to, a binding partner, e.g. another polypeptide, in its natural environment in a cell or an organism and which is preferably capable of

exhibiting a pharmacological activity. The first domain is preferably a non-immunoglobulin polypeptide. The first domain may be a naturally occurring polypeptide or a variant thereof having desired, e.g. increased or reduced, biological activity or a fragment of a naturally occurring polypeptide or a variant thereof. The first domain is preferably selected from the ligand-binding domain of a receptor and a receptor-binding domain of a ligand. The terms „ligand" and „receptor" are understood in this context such that ligands are defined as proteins known to function to bind specifically to receptor molecules. The term „receptor" includes soluble or membrane-anchored receptor proteins having a hydrophobic transmembrane region or a phospholipid anchor. Further, the term „receptor" encompasses carrier proteins as well as hormones, cellular adhesive proteins, lectins, growth factors, enzymes, etc.

In a preferred embodiment of the invention the first domain is a ligand-binding receptor domain comprising the extra-cellular domain of a membrane-anchored receptor or a ligand-binding fragment thereof. The receptor is preferably selected from death receptors, growth factor receptors and cytokine receptors. More preferably, the receptor is selected from CD95 (APO-1; Fas), TRAIL receptors, TNF receptors, VEGF receptors, an interleukin receptor such as IL-15Rα. Most preferably the receptor is CD95, a TRAIL receptor, e.g. the TRAIL receptor-1, the TRAIL receptor-2, the TRAIL receptor-3 or the TRAIL receptor-4 or a TNF receptor, e.g. the TNF receptor-1 or the TNF receptor-2.

In a further embodiment, the first domain is a receptor-binding ligand domain. The ligand is preferably selected from death ligands such as the CD95 ligand, TRAIL, TNF, e.g. TNF-α or TNF-β, growth factors, e.g. VEGF and cytokines, such as interferons or interleukins, e.g. IL-15 or variants thereof.

In a still further embodiment, the fusion protein comprises multiple first domains which may be the same or different. A preferred example of such a

multiple fusion protein is a VEGF Trap fusion protein comprising the second extracellular domain of the VEGF receptor 1 (Flt-1) with the third domain of the VEGF receptor 2 (KDR/FIK-1) and an IgG constant region.

The first domain protein is preferably a mammalian protein, more preferably a human protein. For therapeutic purposes in particular, the use of human proteins is preferred.

The second domain of the fusion protein comprises at least a portion of a constant immunoglobulin domain, e.g. a constant heavy immunoglobulin domain or a constant light immunoglobulin domain. Preferably, the second domain comprises at least a portion of a constant heavy immunoglobulin domain. The constant heavy immunoglobulin domain is preferably an Fc fragment comprising the CH2 and CH3 domain and, optionally, at least a part of the hinge region. The immunoglobulin domain may be an IgG, IgM, IgD or IgE immunoglobulin domain or a modified immunoglobulin domain derived therefrom. Preferably, the second domain comprises at least a portion of a constant IgG immunoglobulin domain. The IgG immunoglobulin domain may be selected from IgG1, IgG2, IgG3 of IgG4 domains or from modified domains such as are described in US 5,925,734. The immunoglobulin domain may exhibit effector functions, particularly effector functions selected from ADCC and/or CDC. In some embodiments, however, modified immunoglobulin domains having modified, e.g. at least partially deleted, effector functions may be used.

Designing the fusion protein of the present invention comprises a selection of the terminal amino acid(s) of the first domain and of the second domain in order to create an at least one amino acid overlap between both domains. In order to achieve this goal it is usually necessary to delete one or several amino acids from a first and/or second domain and/or to add one or several amino acids from the naturally occurring adjacent domain to the first and/or second domain. For example, it may be necessary to provide a first domain having a deletion of preferably up to 10 and, more preferably, up to 6 amino

acids, e.g. 1, 2, 3, 4, 5 or 6 amino acids from naturally occurring domain boundaries. On the other hand, it may be required to add preferably up to 10 and, more preferably, up to 6 amino acids, e.g. 1, 2, 3, 4, 5 or 6 amino acids from a naturally occurring adjacent domain to the first and/or second domain. When deleting and/or adding amino acids, however, one has to take care that the biological activity of the first domain and/or the second domain is not detrimentally affected.

The fusion protein of the invention may comprise an N-terminal signal sequence which allows secretion from a host cell after recombinant expression. The signal sequence may be a signal sequence which is homologous to the first domain of the fusion protein. Alternatively, the signal sequence may also be a heterologous signal sequence, e.g. the Igκ or the Igλ signal peptide sequence. In a different embodiment, the fusion protein is free from an N-terminal sequence, thus representing the mature form of the fusion protein.

The overlap between the first and the second domain or between two first domains has a length of preferably 1, 2 or 3 amino acids. More preferably the overlap has a length of one amino acid. Examples of overlapping amino acids are S, E, K, H, T, P and D.

The present invention is explained in detail below with regard to several specific preferred embodiments. It should be noted, however, that further fusion proteins of the invention may be manufactured by analogous means.

In a first preferred embodiment the first domain is the extracellular domain of human CD95. The extracellular domain of the fusion protein preferably comprises the amino acid sequence up to amino acid 170, 171, 172 or 173 of human CD95. Preferably, the extracellular domain of CD95 is fused with a human IgG Fc fragment, e.g. a human IgG1 Fc fragment. The amino acid sequence of the human CD95 molecule is shown in Figure 1. The amino acid sequence of the human IgG1 chain constant domain is shown in Figure

2. Especially preferred is the fusion protein comprising the amino acid sequence as shown in Figures 3A and 3B, wherein the overlapping amino acid sequence is S.

In a further especially preferred embodiment the first domain is the extracellular domain of a human TRAIL receptor, e.g. the human TRAIL receptor-1, the human TRAIL receptor-2, the human TRAIL receptor-3 and the human TRAIL receptor-4. The extracellular domain preferably comprises the amino acid sequence up to amino acid 232, 233, 234, 235, 236, 237, 238, 239 (TRAILR-1), 204, 205, 206, 207, 208, 209, 210 (TRAILR-2 long), 185, 186, 187, 188, 189, 190, 191 (TRAILR-2 long – without repeat), 179, 180, 181, 182, 183, 184 (TRAILR-2 short), 228, 229, 230, 231, 232, 233, 234, 235, 236, (TRAILR-3), 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161 (TRAILR-3 without repeat) and 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211 (TRAILR-4). Especially preferred is the human TRAIL receptor-2. The extracellular human TRAIL receptor domain may be fused with a human IgG-1 Fc fragment. The amino acid sequences of human TRAIL receptors are shown in Figure 4 (TRAILR-1), Figure 6 (TRAILR-2 long), Figure 9 (TRAILR-2 short), Figure 11 (TRAIL-3) and Figure 14 (TRAILR-4). Specific examples of preferred fusion proteins comprise amino acid sequences as shown in Figure 5, 7, 8, 10, 12, 13 and 15.

In still a further preferred embodiment the fusion protein comprises a first domain which is the extracellular domain of a human TNF receptor, e.g. a human TNF receptor-1 or a human TNF receptor-2. The extracellular domain preferably comprises the amino acid sequence up to amino acid 203, 204, 205, 206, 207, 208, 209, 210, 211 (TNF-R1) or 248, 249, 250, 251, 252, 253, 254, 255, 256, 257 (TNF-R2). The extracellular domain of the human TNF receptor may be fused to a human IgG-1 Fc fragment. The amino acid sequence of human TNF receptors are shown in Figures 16 (TNF-R1) and 18 (TNF-R2). Specific examples of preferred fusion protein comprise amino acid sequences as shown in Figures 17 and 19.

A further aspect of the present invention relates to a nucleic acid molecule encoding a fusion protein as described above. The nucleic acid molecule may be a DNA molecule, e.g. a double-stranded or single-stranded DNA molecule, or an RNA molecule. The nucleic acid molecule may encode the fusion protein or a precursor thereof, e.g. a pro- or pre-proform of the fusion protein which may comprise a signal sequence or other heterologous amino acid portions for secretion or purification which are preferably located at the N- and/or C-terminus of the fusion protein. The heterologous amino acid portions may be linked to the first and/or second domain via a protease cleavage site, e.g. a Factor X_a, thrombin or IgA protease cleavage site.

The nucleic acid molecule may be operatively linked to an expression control sequence, e.g. an expression control sequence which allows expression of the nucleic acid molecule in a desired host cell. The nucleic acid molecule may be located on a vector, e.g. a plasmid, a bacteriophage, a viral vector, a chromosal integration vector, etc. Examples of suitable expression control sequences and vectors are described for example by Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, and Ausubel et al. (1989), *Current Protocols in Molecular Biology*, John Wiley & Sons.

Various expression vector/host cell systems may be used to express the nucleic acid sequences encoding the fusion proteins of the present invention. Suitable host cells include, but are not limited to, prokaryotic cells such as bacteria, e.g. *E.coli*, eukaryotic host cells such as yeast cells, insect cells, plant cells or animal cells, preferably mammalian cells and, more preferably, human cells.

Further, the invention relates to a non-human organism transformed or transfected with a nucleic acid molecule as described above. Such transgenic organisms may be generated by known methods of genetic transfer including homologous recombination.

A further aspect of the present invention relates to a pharmaceutical composition comprising as an active agent at least one fusion protein or a nucleic acid molecule coding thereof as described above. In an especially preferred embodiment, the first domain is a soluble death receptor, e.g. the extracellular domain of a death receptor as described above for use in the prophylaxis and/or treatment of disorders associated with apoptosis. Most preferably, the first domain is the extracellular CD95 domain.

In this embodiment of the invention the composition may be used in the prophylaxis and/or treatment of disorders selected from autoimmune disorders, AIDS, heart disorders, e.g. myocardial infarction, graft-versus-host-disorders, transplant rejection, brain damage, e.g. stroke, spinal cord injuries, e.g. paraplegia, sepsis, hepatitis, disorders associated with inflammation, ischemic reperfusion injury and renal disorders. These disorders and further disorders which may be treated by administration of death receptor fusion proteins, particularly CD95 fusion proteins, are described in WO 95/27735, WO 99/50413, WO 01/41803, EP-A-0 965 637 and EP-A-0 992 243 which are herein incorporated by reference.

The fusion protein is administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means. For example, the fusion protein may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficacy and toxicity may be determined according to standard protocols.The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly or intravenously or locally, e.g. intranasally, subcutaneously or intrathecally. Preferred is intravenous administration.

Especially preferred is a death ligand inhibitor, e.g. a soluble extracellular CD95 or TRAIL receptor domain fused to an Fc fragment.

The dose of the fusion protein administered will of course be dependent on

the subject to be treated, on the subject's weight, the type and severity of the injury, the manner of administration and the judgement of the prescribing physician. For the administration of CD95 or TRAIL-R fusion proteins, a daily dose of 0,001 to 100 mg/kg is suitable.

Moreover, the invention relates to a method for manufacturing a fusion protein comprising

(i) at least one first domain comprising a biologically active protein fused to

(ii) a second domain comprising at least a portion of a constant immunoglobulin domain with reduced immunogenic potential, wherein the first domain is fused to the second domain with at least one amino acid overlap.

Still a further aspect of the present relates to a fusion protein comprising:

(i) at least one first domain comprising a biologically active polypeptide fused to

(ii) a heterologous second domain which is capable of oligomerising the fusion protein wherein there is at least one amino acid overlap between the first and the second domain in the fusion region.

Fusion proteins comprising heterologous second domains which are capable or oligomerising the fusion proteins in the absence of third proteins are described in WO 01/49866 and in WO 02/090553, for example, which are incorporated herein by reference. The presence of at least one amino acid overlap, e.g. one, two or three amino acids overlap, between the first and the second domain in the fusion proteins leads – as explained above – to fusion proteins with reduced immunogenic potential.

The first domain in this oligomerising fusion protein is defined as above. Preferably, the first domain is an extracellular domain of a membrane-anchored receptor, or a ligand-binding fragment thereof. Especially preferred

is that the receptor is selected from CD95, a TRAIL receptor, particularly the TRAIL receptor-2 and a TNF receptor, particularly the TNF receptor-2. Alternatively, the first domain may be a receptor-binding ligand domain, wherein the ligand is preferably selected from CD95 ligand, TRAIL and TNF. Specific examples of preferred first domains are as described above.

The second domain of the fusion protein comprises an oligomerising portion of a protein. Preferably, the second domain is capable of di- tri- tetra- or pentamerising the fusion protein. In this context, particular reference is made to the disclosure of WO 01/49866 and WO 02/090553, which are herein incorporated by reference. Preferred examples of second domains are C1q, MBP (Mannose Binding Protein), SP-A (Lung Surfactant Protein-A), SP-D (Lung Surfactant Protein-D), BC (Bovine Serum Conglutinine), CL43 (Bovine Collectine-43), ACRP-30 (a protein from the C1q family) and COMP (Cartilage Oligomeric Matrix Protein) or the collagen domain of EDA or a functionally active derivative thereof. Especially preferred are portions of ACRP–30, particularly of the human ACRP-30 protein, e.g. amino acids 18 to 108, or 18 to 110 or of COMP.

As described above, the first domain(s) of the fusion protein may be located at the N- or C-terminus and the second domain at the C- or N-terminus. Further, both the first and the second domains are preferably from the same species, more preferably of human origin. Furthermore, the features relating to preferred embodiments of the fusion proteins based on immunoglobulins also apply to the oligomerising fusion proteins.

The reduced immunogenic potential of the fusion protein results from the lack of non-naturally occurring transitions between the first and the second domain in the fusion proteins, which in turn leads to a decreased potential for the formation of neo-epitopes resulting from the fusion between two heterologous polypeptides.

The present invention is illustrated further by the following Figures and Examples.

Figure Legend

Figure 1: the amino acid sequence of the human CD95 (APO-1; Fas) protein;

Figure 2: the amino acid sequence of the human IgG-1 chain C-region;

Figures 3A and 3B: a preferred example of a CD95-Fc IgG1 fusion protein with an overlapping amino acid;

Figure 4: the amino acid sequence of the human TRAIL receptor-1;

Figure 5: preferred examples of TRAILR-1 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 6: the amino acid sequence of human TRAIL receptor-2 (long form);

Figure 7: preferred examples of TRAILR-2 (long) Fc IgG1 fusion proteins with overlapping amino acids, including a repeat sequence;

Figure 8: preferred examples of TRAILR-2 (long form) Fc fusion proteins with overlapping amino acids (without repeat sequence);

Figure 9: the amino acid sequence of human TRAILR-2 (short form);

Figure 10: preferred examples of TRAILR-2 (short) Fc IgG1 fusion proteins with overlapping amino acids;

Figure 11: the amino acid sequence of human TRAIL receptor R-3;

Figure 12: preferred examples of TRAILR-3 Fc IgG1 fusion proteins with overlapping amino acids (repeats included);

Figure 13: preferred examples of TRAILR-3 Fc IgG1 fusion proteins with overlapping amino acids (repeats not included);

Figure 14: the amino acid sequence of human TRAIL receptor-4;

Figure 15: preferred examples of TRAILR-4 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 16: the amino acid sequence of human tumor necrosis factor receptor-1;

Figure 17: preferred examples of TNFR-1 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 18: the amino acid sequence of human tumor necrosis factor

receptor-2;

Figure 19: preferred examples of TNF-R2 Fc IgG1 fusion proteins with overlapping amino acids.

Example 1

Fusion protein consisting of the human CD95 extracellular domain and the human IgG1 Fc domain with overlapping amino acids.

Human CD95 extracellular domain

Bases 221-736 of Human CD95 (Genbank Acc. No. X63717). Utilized Sequence from Oehm, A., "Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumour Necrosis Factor/Nerve Growth Factor Receptor Superfamily," Journal of Biological Chemistry Vol.267, No.15, pp.10709-10715, 1992. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. PCRs were used to amplify the cDNA of the extracellular domain of CD95 by including a restriction Hind III Site and a Kozak Sequence at the 5' of the Extracellular domain and at the 3' a Bgl II site (termination of the extracellular domain).

PCR primers for the amplification of CD95 cDNA with Taq polymerase:
Sense huCD95-Hind III: TATA AAGCTT GCC ACC ATG CTG GGC ATC TG (SEQ ID NO:21)
Antisense huCD95-Bgl II: TATA AGATCT GGA TCC TTC CTC TTT GC (SEQ ID NO:2)

Human IgG1 Fc domain

Sequence: 2050-2745 bp. Sequence used from, Ellison, J., "The nucleotide sequence of human immunoglobulin C gene", Nucleic Acid Research, Volume 10 Number 13, 1982. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. A PCR was used to amplify the cDNA of human IgG1

Fc (partial hinge-CH3) by including a restriction Bgl II site at the 5' of the primer and at the 3' primer after the stop codon, an Xho I site.

PCR primers for the amplification of IgG1 Fc cDNA with Taq polymerase:
Sense hulgG1Fc-BglII: TATA AGATCT TGT GAC AAA ACT CAC ACA TG (SEQ ID NO: 3)
Antisense hulgG1Fc-XhoI: TATA CTCGAG TCA TTT ACC CGG AGA CAG GG (SEQ ID NO: 4)

Cloning Procedure:

Following amplification the IgG1 Fc PCR product was digested with Bgl II and Xho I. The CD95 PCR product was digested with Hind III and Bgl II and pcDNA3.1 (with CMV promoter) with Hind III and Xho I. The products were purified via gel extraction (Qiagen Kit).

The hulgG1Fc and CD95 fragments were ligated with T4 ligase into pcDNA3.1. After transfection of One Shot Top 10 chemically competent cells (E.coli) from Invitrogen Ordering # C4040-10 and amplification, a plasmid preparation was performed with Qiagen Plasmid Prep Kit.

A three point ligation was performed by digesting pcDNA3.1 with HindIII and XhoI, CD95EC with HindIII and BglII, and hulgG1 Fc with BglII and XhoI. The presence of the CD95-hulgG1 Fc insert in pcDNA3.1 was verified by sequencing and restriction enzyme analysis. The vector containing insert was digested with HindIII and XbaI and the insert was ligated into pcDNA3.1 containing the EF-1 promoter.

The Kozak sequence of the original CD95-Fc construct was changed from GCCACCATGC to GCCGCCACCATGG by amplification of the whole CD95-Fc product with the primers SEQ ID 5 and SEQ ID 6.

Primers for Changing the Kozak Sequence from GCCACCATGC to GCCGCCACCATGG:

ShuCD95EC_altKozak TATA AAGCTT GCC GCC ACC ATG GTG GGC ATC (SEQ ID NO. 5)

AS698 hulgG1Fc-Xho1 TATA CTCGAG TCA TTT ACC CGG AGA CAG GG (SEQ ID NO:6)

Cloning Procedure:

The PCR product was cloned in pcDNA3.1/V5 His Topo vector from Invitrogen (Ordering # K4800-01), digested with Hind III and Xba I as well as pcDNA3.1 containing the pEF promoter and ligated with T4 Ligase.

Expression and Isolation

The construct encoding the final product was transfected into cell lines suitable for protein expression. Transfection can be performed by any standard method know to those skilled in the art. Examples include electroporation, liposomal mediated transfer, calcium phosphate transfection. Cell lines suitable for the expression include 293T cells, COS-1, COS-7 and CHO cells. Other cell lines may be used.

In this example, 293T cells were transiently transfected by the calcium phosphate method. Alternatively, CHO cells were transfected utilizing FuGene6 and stable clones were selected.

The desired protein can be purified from the cell culture medium by chromatographic methods. Methods include but are not limited to affinity chromatography on protein-G or protein-A columns, ion-exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatograpy or a combination of these methods.

In the example the supernatant was purified on IgG columns (Amersham Pharmacia) according to the manufacturers instructions, leading to a higly purified product in a single step.

Example 2.

Fusion protein consisting of the TRAIL receptor-2 and the human IgG1 Fc domain with overlapping amino acids

Human IgG1 Fc domain:

Sequence used from, Ellison, J., "The nucleotide sequence of human immunoglobulin C gene", Nucleic Acid Research, Volume 10 Number 13, 1982. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. A PCR was used to amplify the cDNA of human IgG1 Fc (partial hinge-CH3) with an overlapping sequence to TRAILR2 at the 5' end and at the 3' end after the stop codon an EcoRI site.

I. Primer: **Sense_huIgG1** (SEQ ID NO: 7)
cca ggg act cct gcc TCT TGT GAC AAA ACT CAC ACA TG (Capital letters => part of huIgG1)

II. Primer: **Antisense_ERIhuIgG1** (SEQ ID NO: 8)
TATA gaa ttc tca ttt acc cgg aga cag gg

TRAILR2:

Utilized Sequence from Walczak H., "TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL" The EMBO Journal Vol.16, No.17, pp.5386-5397, 1997. (Accession number DDBJ/EMBL/GenBank: AF016849) cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using an Oligo dT primer. A PCR was used to amplify the cDNA of TRAILR2 domain by including a restriction site Hind III and a Kozak Sequence at the 5' end and at the 3' end an overlapping sequence to human IgG1.

III. Primer: **Sense_HIII_TRAILR2** (SEQ ID NO: 9)
TATA aag ctt gcc gcc acc atg gaa caa cgg gga cag aac

IV. Primer: **Antisense_TRAILR2** (SEQ ID NO: 10)
gtg agt ttt gtc aca aga GGC AGG AGT CCC TGG (Capital letters => part huTRAIL-R2, in reverse)

Cloning Procedure:
Following the amplification a gel extraction was performed to isolate the modified inserts. Then a third PCR utilizing both fragments was performed. Due to the overlap of both fragments and the primers at the end, this PCR joins in one product. Afterwards the product was digested with Hind III and EcoR I and ligated in a suitable expression vector, e.g. pcDNA3.1 (Invitrogen).

III. Primer: **Sense_HIII_TRAILR2** (SEQ ID NO: 11)
TATA aag ctt gcc gcc acc atg gaa caa cgg gga cag aac

II. Primer: **Antisense_ERIhuIgG1** (SEQ ID NO: 12)
TATA gaa ttc tca ttt acc cgg aga cag gg

Expression
The construct was cloned and expressed in suitable host cells as described in Example 1.

Example 3.

Use of a CD95-Fc construct for the regeneration and functional recovery after spinal cord injury.

The CD95-Fc construct with overlapping amino acids as described in Example 1 was used for the treatment of spinal cord-injury in a mouse model as described by Demjen et al., *Nat Med.* (March 7, 2004). It was found that administration of the construct promotes regeneration and functional recovery after spinal cord injury.

Example 4.

Use of CD95-Fc construct for the attenuation of brain damage in stroke.

The CD95-Fc construct with overlapping amino acids was investigated for its influence on primary ischemic death and secondary inflammatory injury in a mouse model as described by Martin-Villalba et al. (*Cell Death Differ.* 8 (2001), 679-686). It was found that administration of the CD95-Fc construct resulted in a significant decrease in both infarct volumes and mortality.

Claims

1. A fusion protein comprising
(i) at least one first domain comprising a biologically active polypeptide fused to
(ii) a heterologous second domain comprising at least a portion of a constant immunoglobulin domain wherein there is at least one amino acid overlap between the first domain and the second domain in the fusion region.

2. The fusion protein of claim 1, wherein the first domain is selected from a ligand-binding domain of a receptor and a receptor-binding domain of a ligand.

3. The fusion protein of claims 1 or 2, wherein the first domain is a ligand-binding receptor domain comprising an extracellular domain of a membrane-anchored receptor or a ligand-binding fragment thereof.

4. The fusion protein of claims 2 or 3, wherein the receptor is selected from death receptors, growth factor receptors and cytokine receptors.

5. The fusion protein of claim 4, wherein the receptor is selected from CD95, a TRAIL receptor, a TNF receptor and a VEGF receptor.

6. The fusion protein of claims 1 or 2, wherein the first domain is a receptor-binding ligand domain.

7. The fusion protein of claims 1 or 6, wherein the ligand is selected from death ligands, growth factors and cytokines.

8. The fusion protein of claim 7, wherein the ligand is selected from CD95 ligand, TRAIL, TNF, VEGF and IL-15.

9. The fusion protein of any one of claims 1 to 8 wherein the at least one first domain is derived from a human protein.

10. The fusion protein of any one of claims 1 to 9, wherein the second domain comprises at least a portion of a constant heavy immunoglobulin domain.

11. The fusion protein of any one of claims 1 to 9, wherein the second domain is an Fc fragment of a constant heavy immunoglobulin domain comprising the CH2 and CH3 domain and optionally at least a part of the hinge region.

12. The fusion protein of any one of claims 1 to 11, wherein the second domain comprises at least a portion of a constant IgG immunoglobulin domain.

13. The fusion protein of any one of claims 1 to 12, wherein the second domain comprises at least a portion of a constant IgG1, IgG2, IgG3 or IgG4 immunoglobulin domain or a variant thereof.

14. The fusion protein of any one of claims 1 to 13 wherein the immunoglobulin domain exhibits effector functions, particularly effector functions selected from ADCC and/or CDC.

15. The fusion protein of any one of claims 1 to 14, wherein the second domain is derived from a human immunoglobulin.

16. The fusion protein of any one of claims 1 to 15 wherein the overlap has a length of 1, 2 or 3 amino acids.

17. The fusion protein of any one of claims 1 to 16 wherein at least one carboxy terminal amino acid of the first domain overlaps with at least one amino terminal amino acid of the second domain.

18. The fusion protein of any one of claims 1 to 17 wherein the fusion region is free from a non-naturally occurring transition between the last amino acid of one domain and the first amino acid of the other domain.

19. The fusion protein of any one of claims 1 to 18 wherein the first domain and/or second domain comprises a deletion of preferably up to 6 amino acids.

20. The fusion protein of any one of claims 1 to 19 wherein the first domain and/or second domain comprises an addition of preferably up to 6 amino acids.

21. The fusion protein of any one of claims 1 to 20 which comprises an N-terminal signal sequence.

22. The fusion protein of any one of claims 1 to 20 which lacks an N-terminal signal sequence.

23. The fusion protein of any one of claims 1 to 22 wherein the overlapping amino acid sequence is selected from S, E, K, H, T, P and D.

24. The fusion protein of any one of claims 1 to 23 wherein the first domain is the extracellular domain of human CD95.

25. The fusion protein of claim 24 wherein the extracellular domain of CD95 has the amino acid sequence up to amino acid 170, 171, 172 or 173 of human CD 95.

26. The fusion protein of claim 25 comprising an amino acid sequence as shown in Figures 3A and 3B.

27. The fusion protein of any one of claims 1 to 23 wherein the first domain is the extracellular domain of a human TRAIL receptor.

28. The fusion protein of claim 22, wherein the human TRAIL receptor is selected from human TRAIL receptor-1, human TRAIL receptor-2, human TRAIL receptor-3 and human TRAIL receptor-4.

29. The fusion protein of claim 28 comprising an amino acid sequence as shown in Figures 5, 7, 8, 10, 12, 13 or 15.

30. The fusion protein of any one of claims 1 to 23 wherein the first domain is the extracellular domain of a human TNF receptor.

31. The fusion protein of claim 30, wherein the human TNF receptor is selected from human TNF receptor-1 and human TNF receptor-2.

32. The fusion protein of claim 31 comprising the amino acid sequence as shown in Figures 17 or 19.

33. A nucleic acid molecule encoding a fusion protein of any one of claims 1 to 32 or a precursor thereof.

34. The nucleic acid molecule of claim 33 which is operatively linked to an expression control sequence.

35. The nucleic acid molecule of claims 33 or 34 which is located on a vector.

36. A cell transformed or transfected with a nucleic acid molecule of any one of claims 33 to 35.

37. The cell of claim 36 which is a prokaryotic cell.

38. The cell of claim 37 which is a eukaryotic cell, preferably a mammalian cell and more preferably a human cell.

39. A non-human organism transformed or transfected with a nucleic acid molecule of any one of claims 33 to 35.

40. A pharmaceutical composition comprising as an active agent a fusion protein of any one of claims 1 to 32 or a nucleic acid molecule of any one of claims 33 to 35.

41. The composition of claim 40 wherein the first domain is a soluble death receptor for use in the prophylaxis and/or treatment of disorders associated with apoptosis.

42. The composition of claim 41 wherein the first domain is the extracellular CD95 domain.

43. The composition of claims 41 or 42 for use in the prophylaxis and/or treatment of disorders selected from autoimmune disorders, AIDS, heart disorders, e.g. myocardial infarction, graft-versus-host-disorders, e.g. transplant rejection, spinal cord injuries, e.g. paraplegia, sepsis, hepatitis, disorders associated with inflammation, ischemic reperfusion injury and renal disorders.

44. A method for manufacturing a fusion protein comprising

(i) at least one first domain comprising a biologically active polypeptide fused to

(ii) a second domain comprising at least a portion of a constant immunoglobulin domain with reduced immunogenic potential, wherein the first domain is fused to the second domain with at least one amino acid overlap.

45. A fusion protein comprising
(i) at least one first domain comprising a biologically active polypeptide fused to
(ii) a heterologous second domain which is capable of oligomerising the fusion protein wherein there is at least one amino acid overlap between the first and the second domain in the fusion region.

46. The fusion protein of claim 45, wherein the first domain comprises an extracellular domain of a membrane-anchored receptor or a ligand-binding fragment thereof.

47. The fusion protein of claim 46, wherein the receptor is selected from CD95, a TRAIL receptor and a TNF receptor.

48. The fusion protein of claim 48, wherein the first domain comprises a receptor-binding ligand domain.

49. The fusion protein of claim 48, wherein the ligand is selected from CD95 ligand, TRAIL and TNF.

50. The fusion protein of any one of claims 45 to 49, wherein the second domain comprises an oligomerising portion of a protein selected from C1q, MBP, SP-A, SP-D, BC, CL43 and ACRP30 and COMP or the collagen domain of EDA or a functionally active derivative thereof.

51. The fusion protein of any one of claims 45 to 50, wherein the second domain is capable of di-, tri-, tetra- or pentamerising the fusion protein.

Figure 1

CD95

>sp|P25445|TNR6_HUMAN Tumor necrosis factor receptor superfamily member 6 precursor (FASL receptor) (Apoptosis-mediating surface antigen FAS) (Apo-1 antigen) (CD95) - Homo sapiens (Human).

```
1                                                                    60
MLGIWTLLPL VLTSVARLSS KSVNAQVTDI NSKGLELRKT VTTVETQNLE GLHHDGQFCH
61                                                                  120
KPCPPGERKA RDCTVNGDEP DCVPCQEGKE YTDKAHFSSK CRRCRLCDEG HGLEVEINCT
121                                                                 180
RTQNTKCRCK PNFFCNSTVC EHCDPCTKCE HGIIKECTLT SNTKCKEEGS RSNLGWLCLL
181                                                                 240
LLPIPLIVWV KRKEVQKTCR KHRKENQGSH ESPTLNPETV AINLSDVDLS KYITTIAGVM
241                                                                 300
TLSQVKGFVR KNGVNEAKID EIKNDNVQDT AEQKVQLLRN WHQLHGKKEA YDTLIKDLKK
301                                335
ANLCTLAEKI QTIILKDITS DSENSNFRNE IQSLV
```

AA 1-16 Signal peptide (potential)
AA 17-173 extracellular domain (potential)
AA 47-83 CRD1
AA 84-127 CRD2.
AA 128-166 CRD3
AA 174-190 transmembrane (potential)
AA 191-335 cytoplasmic (potential)

Figure 2

IgG1

>sp|P01857|GC1_HUMAN Ig gamma-1 chain C region - Homo sapiens (Human).

```
1                                                                    60
ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
61                                                                  120
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG
121                                                                 180
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
181                                                                 240
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE
241                                                                 300
LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
301                           330
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
```

AA 99-110 hinge region
AA 111-223 CH2 region
AA 224-330 CH3 region
Variants D239E, L241M

Figure 3A

CD95-Fc (AA 1-172 CD95 and AA 102-330 IgG1)

```
1                                                            60
MLGIWTLLPL VLTSVARLSS KSVNAQVTDI NSKGLELRKT VTTVETQNLE GLHHDGQFCH
61                                                          120
KPCPPGERKA RDCTVNGDEP DCVPCQEGKE YTDKAHFSSK CRRCRLCDEG HGLEVEINCT
121                                                         180
RTQNTKCRCK PNFFCNSTVC EHCDPCTKCE HGIIKECTLT SNTKCKEEGS RSCDKTHTCP
181                                                         240
PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA
241                                                         300
KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
301                                                         360
VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY
361                                        400
SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
```

Figure 3B

Example of a preferred CD59-Fc fusion protein with an overlapping amino acid:

CD95 extracellular domain	huIgG1
131 173 PNFFCNSTVC EHCDPCTKCE HGIIKECTLT SNTKCKEEGS RSN	99 120 EP KSCDKTHTCP PCPAPELLGG
PNFFCNSTVC EHCDPCTKCE HGIIKECTLT SNTKCKEEGS RSCDKTHTCP PCPAPELLGG	

Figure 4

3. TRAIL-R1

>sp|O00220|T10A_HUMAN Tumor necrosis factor receptor superfamily member 10A precursor (Death receptor 4) (TNF-related apoptosis-inducing ligand receptor 1) (TRAIL receptor-1) (TRAIL-R1) - Homo sapiens (Human).

```
1                                                                    60
MAPPPARVHL GAFLAVTPNP GSAASGTEAA AATPSKVWGS SAGRIEPRGG GRGALPTSMG
61                                                                  120
QHGPSARARA GRAPGPRPAR EASPRLRVHK TFKFVVVGVL LQVVPSSAAT IKLHDQSIGT
121                                                                 180
QQWEHSPLGE LCPPGSHRSE HPGACNRCTE GVGYTNASNN LFACLPCTAC KSDEEERSPC
181                                                                 240
TTTRNTACQC KPGTFRNDNS AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHNI
241                                                                 300
WVILVVTLVV PLLLVAVLIV CCCIGSGCGG DPKCMDRVCF WRLGLLRGPG AEDNAHNEIL
301                                                                 360
SNADSLSTFV SEQQMESQEP ADLTGVTVQS PGEAQCLLGP AEAEGSQRRR LLVPANGADP
361                                                                 420
TETLMLFFDK FANIVPFDSW DQLMRQLDLT KNEIDVVRAG TAGPGDALYA MLMKWVNKTG
421                                                  468
RNASIHTLLD ALERMEERHA KEKIQDLLVD SGKFIYLEDG TGSAVSLE
```

AA 1-23 Signal peptide (potential)
AA 24-239 extracellular domain (potential)
AA 107-145 CRD1
AA 147-188 CRD2
AA 189-229 CRD3
AA 240-262 transmembrane (potential)
AA 263-468 cytoplasmic (potential)

Figure 5

Examples of Trail-R1-Fc fusion proteins with overlapping amino acids:

Trail R1 extracellular domain	huIgG1
201 239 AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHN	99 120 EP KSCDKTHTCP PCPAPELLGG
AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKEP KSCDKTHTCP PCPAPELLGG	
201 239 AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHN	99 120 EP KSCDKTHTCP PCPAPELLGG
AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKSCDKTHTCP PCPAPELLGG	
201 239 AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHN	99 120 EP KSCDKTHTCP PCPAPELLGG
AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESCDKTHTCP PCPAPELLGG	
201 239 AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHN	99 120 EP KSCDKTHTCP PCPAPELLGG
AEMCRKCSRG CPRGMVKVKD CTPWSDIECV HKESGNGHTCP PCPAPELLGG	

Figure 6

4. TRAIL-R2 (long)

>sp|O14763|T10B_HUMAN Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor-2) (TRAIL-R2) - Homo sapiens (Human).

```
1                                                                    60
MEQRGQNAPA ASGARKRHGP GPREARGARP GPRVPKTLVL VVAAVLLLVS AESALITQQD
61                                                                  120
LAPQQRAAPQ QKRSSPSEGL CPPGHHISED GRDCISCKYG QDYSTHWNDL LFCLRCTRCD
121                                                                 180
SGEVELSPCT TTRNTVCQCE EGTFREEDSP EMCRKCRTGC PRGMVKVGDC TPWSDIECVH
181                                                                 240
KESGTKHSGE APAVEETVTS SPGTPASPCS LSGIIIGVTV AAVVLIVAVF VCKSLLWKKV
241                                                                 300
LPYLKGICSG GGGDPERVDR SSQRPGAEDN VLNEIVSILQ PTQVPEQEME VQEPAEPTGV
301                                                                 360
NMLSPGESEH LLEPAEAERS QRRRLLVPAN EGDPTETLRQ CFDDFADLVP FDSWEPLMRK
361                                                                 420
LGLMDNEIKV AKAEAAGHRD TLYTMLIKWV NKTGRDASVH TLLDALETLG ERLAKQKIED
421                440
HLLSSGKFMY LEGNADSAMS
```

AA 1-55 Signal peptide
AA 56-210 extracellular domain (potential)
AA 57-94 CRD1
AA 97-137 CRD2
AA 138-178 CRD3
AA 192-206 TAPE
AA 211-231 transmembrane (potential)
AA 232-440 cytoplasmic (potential)

Figure 7

Examples of Trail-R2(long)-Fc fusion proteins with overlapping amino acids ("repeat" included):

Trail R2 (long) extracellular domain	huIgG1
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCSCDKTHTCP PCPAPELLGG Bevorzugte Ausführung (wie in EP 03006949.6 beschrieben)	
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASCDKTHTCP PCPAPELLGG	
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASP KSCDKTHTCP PCPAPELLGG	
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTP KSCDKTHTCP PCPAPELLGG	
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCDKTHTCP PCPAPELLGG	
171 210 TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTPASPCS	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGE APAVEETVTS SPGTHTCP PCPAPELLGG	

Figure 8

Examples of Trail-R2(long)-Fc fusion proteins with overlapping amino acids ("repeat" not included):

Trail R2 (long) extracellular domain	huIgG1
171 191 TPWSDIECVH KESGTKHSGE A	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSGEP KSCDKTHTCP PCPAPELLGG	
171 191 TPWSDIECVH KESGTKHSGE A	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKSCDKTHTCP PCPAPELLGG	
171 191 TPWSDIECVH KESGTKHSGE A	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHSCDKTHTCP PCPAPELLGG	
171 191 TPWSDIECVH KESGTKHSGE A	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTHTCP PCPAPELLGG	
171 191 TPWSDIECVH KESGTKHSGE A	99 120 EP KSCDKTHTCP PCPAPELLGG
TPWSDIECVH KESGTKHTCP PCPAPELLGG	

Figure 9

5. TRAIL-R2 (short)

>sp|O14763|T10B_HUMAN Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor-2) (TRAIL-R2) - Homo sapiens (Human).

```
1                                                                    60
MEQRGQNAPA ASGARKRHGP GPREARGARP GPRVPKTLVL VVAAVLLLVS AESALITQQD
61                                                                  120
LAPQQRAAPQ QKRSSPSEGL CPPGHHISED GRDCISCKYG QDYSTHWNDL LFCLRCTRCD
121                                                                 180
SGEVELSPCT TTRNTVCQCE EGTFREEDSP EMCRKCRTGC PRGMVKVGDC TPWSDIECVH
181                                                                 240
KESGIIIGVT VAAVVLIVAV FVCKSLLWKK VLPYLKGICS GGGGDPERVD RSSQRPGAED
241                                                                 300
NVLNEIVSIL QPTQVPEQEM EVQEPAEPTG VNMLSPGESE HLLEPAEAER SQRRRLLVPA
301                                                                 360
NEGDPTETLR QCFDDFADLV PFDSWEPLMR KLGLMDNEIK VAKAEAAGHR DTLYTMLIKW
361                                                            411
VNKTGRDASV HTLLDALETL GERLAKQKIE DHLLSSGKFM YLEGNADSAM S
```

AA 1-55 Signal peptide
AA 56-184 extracellular domain (potential)
AA 57-94 CRD1
AA 97-137 CRD2
AA 138-178 CRD3
AA 213-202 transmembrane (potential)
AA 203-411 cytoplasmic (potential)

Figure 10

Examples of Trail-R2(short)-Fc fusion proteins with overlapping amino acids:

Trail-R2 (short) extracellular domain	huIgG1
151 184 EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KESG	99 120 EP KSCDKTHTCP PCPAPELLGG
EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KEP KSCDKTHTCP PCPAPELLGG	
151 184 EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KESG	99 120 EP KSCDKTHTCP PCPAPELLGG
EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KSCDKTHTCP PCPAPELLGG	
151 184 EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KESG	99 120 EP KSCDKTHTCP PCPAPELLGG
EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KESCDKTHTCP PCPAPELLGG	
151 184 EMCRKCRTGC PRGMVKVGDC TPWSDIECVH KESG	99 120 EP KSCDKTHTCP PCPAPELLGG
EMCRKCRTGC PRGMVKVGDC TPWSDIECVHTCP PCPAPELLGG	

Figure 11

6. TRAIL-R3

>sp|O14798|T10C_HUMAN Tumor necrosis factor receptor superfamily member 10C precursor (Decoy receptor 1) (DcR1) (Decoy TRAIL receptor without death domain) (TNF- related apoptosis-inducing ligand receptor 3) (TRAIL receptor-3) (TRAIL-R3) (Trail receptor w

```
1                                                                    60
MARIPKTLKF VVVIVAVLLP VLAYSATTAR QEEVPQQTVA PQQQRHSFKG EECPAGSHRS
61                                                                  120
EHTGACNPCT EGVDYTNASN NEPSCFPCTV CKSDQKHKSS CTMTRDTVCQ CKEGTFRNEN
121                                                                 180
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE TPAAEETMNT SPGTPAPAAE
181                                                                 240
ETMNTSPGTP APAAEETMTT SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPASSHY
241              259
LSCTIVGIIV LIVLLIVFV
```

AA 1-23 Signal peptide
AA 24-236 extracellular domain
AA 29-66 CRD1
AA 69-109 CRD2
AA 110-149 CRD3
AA 162-236 5 x 15 AA tandem tape repeats
AA 237-259 removed in mature form (potential)

Figure 12

Examples of Trail-R3-Fc fusion proteins with overlapping amino acids ("repeats" included):

Trail-R3 extracellular domain	huIgG1
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTP KSCDKTHTCP PCPAPELLGG	
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTT SP KSCDKTHTCP PCPAPELLGG	
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTT SCDKTHTCP PCPAPELLGG	
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTHTCP PCPAPELLGG	
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTTHTCP PCPAPELLGG	
201 236 SPGTPAPAAE ETMTTSPGTP APAAEETMTT SPGTPA	99 120 EP KSCDKTHTCP PCPAPELLGG
SPGTPAPAAE ETMTTSPGTP APAAEETMTHTCP PCPAPELLGG	

Figure 13

Examples of Trail-R3-Fc fusion proteins with overlapping amino acids ("repeats" not included):

Trail-R3 extracellular domain	huIgG1
121 161 SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE T	99 120 EP KSCDKTHTCP PCPAPELLGG
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVEP KSCDKTHTCP PCPAPELLGG	
121 161 SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE T	99 120 EP KSCDKTHTCP PCPAPELLGG
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEP KSCDKTHTCP PCPAPELLGG	
121 161 SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE T	99 120 EP KSCDKTHTCP PCPAPELLGG
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EP KSCDKTHTCP PCPAPELLGG	
121 161 SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE T	99 120 EP KSCDKTHTCP PCPAPELLGG
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE HTCP PCPAPELLGG	
121 161 SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATVE T	99 120 EP KSCDKTHTCP PCPAPELLGG
SPEMCRKCSR CPSGEVQVSN CTSWDDIQCV EEFGANATHTCP PCPAPELLGG	

Figure 14

7. TRAIL-R4

>sp|Q9UBN6|T10D_HUMAN Tumor necrosis factor receptor superfamily member 10D precursor (Decoy receptor 2) (DcR2) (TNF-related apoptosis-inducing ligand receptor 4) (TRAIL receptor-4) (TRAIL-R4) (TRAIL receptor with a truncated death domain) - Homo sapiens

```
1                                                                    60
MGLWGQSVPT ASSARAGRYP GARTASGTRP WLLDPKILKF VVFIVAVLLP VRVDSATIPR
61                                                                  120
QDEVPQQTVA PQQQRRSLKE EECPAGSHRS EYTGACNPCT EGVDYTIASN NLPSCLLCTV
121                                                                 180
CKSGQTNKSS CTTTRDTVCQ CEKGSFQDKN SPEMCRTCRT GCPRGMVKVS NCTPRSDIKC
181                                                                 240
KNESAASSTG KTPAAEETVT TILGMLASPY HYLIIIVVLV IILAVVVVGF SCRKKFISYL
241                                                                 300
KGICSGGGGG PERVHRVLFR RRSCPSRVPG AEDNARNETL SNRYLQPTQV SEQEIQGQEL
301                                                                 360
AELTGVTVES PEEPQRLLEQ AEAEGCQRRR LLVPVNDADS ADISTLLDAS ATLEEGHAKE
361                        386
TIQDQLVGSE KLFYEEDEAG SATSCL
```

AA 1-55 signal peptide
AA 56-211 extracellular domain (potential)
AA 58-97 CRD1
AA 98-139 CRD2
AA 140-180 CRD3
AA 212-232 transmembrane (potential)
AA 233-386 cytoplasmic (potential)

Figure 15

Examples of Trail-R4-Fc fusion proteins with overlapping amino acids:

Trail-R4 extracellular domain	huIgG1
171 211 NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASPY H	99 120 EP KSCDKTHTCP PCPAPELLGG
NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASP KSCDKTHTCP PCPAPELLGG	
171 211 NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASPY H	99 120 EP KSCDKTHTCP PCPAPELLGG
NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASCDKTHTCP PCPAPELLGG	
171 211 NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASPY H	99 120 EP KSCDKTHTCP PCPAPELLGG
NCTPRSDIKC KNESAASSTG KTPAAEETVT THTCP PCPAPELLGG	
171 211 NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASPY H	99 120 EP KSCDKTHTCP PCPAPELLGG
NCTPRSDIKC KNESAASSTG KTPAAEETVT TILGMLASPY HTCP PCPAPELLGG	

Figure 16

1. TNF-R1

>sp|P19438|TR1A_HUMAN Tumor necrosis factor receptor superfamily member 1A precursor (p60) (TNF-R1) (TNF-RI) (p55) (CD120a) [Contains: Tumor necrosis factor binding protein 1 (TBPI)] - Homo sapiens (Human).

```
1                                                                    60
MGLSTVPDLL LPLVLLELLV GIYPSGVIGL VPHLGDREKR DSVCPQGKYI HPQNNSICCT
61                                                                  120
KCHKGTYLYN DCPGPGQDTD CRECESGSFT ASENHLRHCL SCSKCRKEMG QVEISSCTVD
121                                                                 180
RDTVCGCRKN QYRHYWSENL FQCFNCSLCL NGTVHLSCQE KQNTVCTCHA GFFLRENECV
181                                                                 240
SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT TVLLPLVIFF GLCLLSLLFI GLMYRYQRWK
241                                                                 300
SKLYSIVCGK STPEKEGELE GTTTKPLAPN PSFSPTPGFT PTLGFSPVPS STFTSSSTYT
301                                                                 360
PGDCPNFAAP RREVAPPYQG ADPILATALA SDPIPNPLQK WEDSAHKPQS LDTDDPATLY
361                                                                 420
AVVENVPPLR WKEFVRRLGL SDHEIDRLEL QNGRCLREAQ YSMLATWRRR TPRREATLEL
421                                 455
LGRVLRDMDL LGCLEDIEEA LCGPAALPPA PSLLR
```

AA 1-21 Signal peptide
AA 22-211 extracellular domain (potential)
AA 43-82 CRD1
AA 83-125 CRD2
AA 126-166 CRD3
AA 167-196 CRD4
AA 212-234 transmembrane (potential)
AA 235-455 cytoplasmic (potential)

Figure 17

Examples of TNF-R1-Fc fusion proteins with overlapping amino acids:

TNF-R1 extracellular domain	huIgG1
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEP KSCDKTHTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKSCDKTHTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKTHTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSCDKTHTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDKTHTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT THTCP PCPAPELLGG	
171 211 GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT T	99 120 EP KSCDKTHTCP PCPAPELLGG
GFFLRENECV SCSNCKKSLE CTKLCLPQIE NVKGTEDSGTHTCP PCPAPELLGG	

Figure 18

2. TNF-R2

>sp|P20333|TR1B_HUMAN Tumor necrosis factor receptor superfamily member 1B precursor (Tumor necrosis factor receptor 2) (p80) (TNF-R2) (p75) (CD120b) (Etanercept) [Contains: Tumor necrosis factor binding protein 2 (TBPII)] - Homo sapiens (Human).

```
1                                                                    60
MAPVAVWAAL AVGLELWAAA HALPAQVAFT PYAPEPGSTC RLREYYDQTA QMCCSKCSPG
61                                                                   120
QHAKVFCTKT SDTVCDSCED STYTQLWNWV PECLSCGSRC SSDQVETQAC TREQNRICTC
121                                                                  180
RPGWYCALSK QEGCRLCAPL RKCRPGFGVA RPGTETSDVV CKPCAPGTFS NTTSSTDICR
181                                                                  240
PHQICNVVAI PGNASMDAVC TSTSPTRSMA PGAVHLPQPV STRSQHTQPT PEPSTAPSTS
241                                                                  300
FLLPMGPSPP AEGSTGDFAL PVGLIVGVTA LGLLIIGVVN CVIMTQVKKK PLCLQREAKV
301                                                                  360
PHLPADKARG TQGPEQQHLL ITAPSSSSSS LESSASALDR RAPTRNQPQA PGVEASGAGE
361                                                                  420
ARASTGSSDS SPGGHGTQVN VTCIVNVCSS SDHSSQCSSQ ASSTMGDTDS SPSESPKDEQ
421                                          461
VPFSKEECAF RSQLETPETL LGSTEEKPLP LGVPDAGMKP S
```

AA 1-22 Signal peptide
AA 23-257 extracellular domain (potential)
AA 39-76 CRD1
AA 77-118 CRD2
AA 119-162 CRD3
AA 163-201 CRD4
AA 258-287 transmembrane (potential)
AA 288-461 cytoplasmic (potential)

Figure 19

Examples of TNF-R2-Fc fusion proteins with overlapping amino acids:

TNF-R2 extracellular domain	huIgG1
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEP KSCDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSPP KSCDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSP KSCDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSCDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSCDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGDKTHTCP PCPAPELLGG	
221 257 STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTGD	99 120 EP KSCDKTHTCP PCPAPELLGG
STRSQHTQPT PEPSTAPSTS FLLPMGPSPP AEGSTHTCP PCPAPELLGG	